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In Vitro and *in Vivo* Analysis of Cellular Origin of Cervical Squamous Metaplasia

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We have previously shown that cultured normal human endocervical cells (HENs) form epithelium resembling squamous metaplasia in vivo. To analyze the cellular origin of squamous metaplasia, the cytokeratin and mucin expression and morphological features of HENs in monolayer cultures and in implants beneath the skin of nude mice were examined. Primary HENs had two distinct morphological phenotypes in vitro: pleomorphic epithelial cells and keratinocytelike cells. Using a panel of monoclonal antibodies for various cytokeratins (CKs), we observed that the pleomorphic cells, which were the primary outgrowths, expressed CK7 and CK18 and produced mucin, suggesting their origin to be the mucosecretory columnar cells (CCs) of the endocervix. Keratinocytelike cells were observed in proximity of the CC-like cells after a few days of HEN culture. Interestingly, these cells were homogeneously negative for CK7 expression, as for native reserve cells (RCs), and homogeneously positive for CK13 expression with the antibody that is specific for RCs. During early passages, the culture consisted mostly of the RC-like keratinocytelike cells, and in the late passages, the CC-like cells were predominant. HEN implants in nude mice morphologically formed epithelia similar to immature squamous metaplasia and showed variable CK18 expression. Moreover, they showed homogeneous CK13 expression throughout all layers and expressed mucin and CK7 in the suprabasal cells. The possibility that the HEN culture was originally a mixed population of CCs and RCs, that we failed to detect, cannot be eliminated. Our results support the more likely view that the endocervical simple epithelia, which form squamous metaplasia, are bipotential cells and undergo differentiation readily and reversibly to give rise to CC-like and RC-like cells in culture. (Am J Pathol 1993, 143:1150–1158)

The process of epithelial transdifferentiation involves the transformation of one differentiated epithelial phenotype into another.[1] This process is especially common in the human uterine cervix. The normal uterine cervix consists of an endocervical canal lined with a single layer of columnar mucosecretory cells and an outer ectocervix covered by a stratified nonkeratinizing squamous epithelium. As a result of epithelial transdifferentiation, termed squamous metaplasia, the endocervical simple epithelium is replaced by an immature squamous epithelium that progresses into mature squamous epithelium.[2,3] This squamous metaplastic area or transformation zone is the frequent site for the development of cervical intraepithelial neoplasia (CIN) and cervical carcinoma.[2,3]

In vitro morphological evidence of squamous metaplasia has been documented in the progressive changes in the columnar epithelium of long-term organ culture studies using endocervical tissue.[4,5] Moreover, endocervical epithelial cells exhibit some characteristics of the squamous phenotype, *in vitro*, possibly an effect of culturing these cells in serum-free monolayer culture.[6] Recently, we have cultured human endo- and ectocervical epithelial cells (HENs and HECs, respectively)[7] and have developed an HEN/HEC *in vivo* implantation system.[8] When HECs

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are implanted into nude mice, they form a well-differentiated stratified squamous epithelium.[8,9] In contrast, the implanted HENs form metaplastic stratified epithelium.[8] The squamous morphology of this experimental HEN metaplasia suggested that *in vitro/in vivo* transformation can occur, similar to the suspected transformation of endocervical epithelium into squamous metaplasia *in situ*. The aim of the present study was to use the HEN culture/implantation system to examine the cellular origin of cervical squamous metaplasia. The study focused on the two cell types of the endocervix, the cytokeratin (CK) 7-expressing mucosecretory columnar cells (CCs) and the reserve cells (RCs).

The RCs have been accepted as being the source of cervical squamous metaplasia, but to our knowledge, only on the basis of morphological and/or immunohistochemical *in situ* observations.[2,3,10-15] In addition, the nature and origin of RCs have been controversial and remain obscure. CKs, the major class of epithelial intermediate filaments, are markers that have been used to determine differentiation-dependent epithelial cell origin.[16] In recent years, there have been a number of studies of CK expression in normal endocervical epithelium,[12-19] and there is now a general consensus for the CK phenotypes of CCs and RCs. In this report, the detailed CK expression, morphology, and mucin production of HENs in serum-free monolayer culture and in tissues reconstituted in nude mice were investigated. The results with this experimental system suggest that, whereas there may have been two distinct populations of CCs and RCs, more likely the endocervical cells, that adopt a stratified differentiation pattern and form squamous metaplasia under certain conditions, have bipotentiality and undergo differentiation to alternate between CC-like or RC-like HENs in culture.

Materials and Methods

Cell Culture

Human cervical epithelial cell cultures were derived from cervical specimens obtained from hysterectomies performed for benign conditions that had been shown to be free of CIN. The ages of the patients varied between 35 and 57 years. The squamous metaplastic cells of the transformation zone were identified microscopically and excised at a large distance from the endo- and ectocervical samples. HEN and HEC cultures were initiated from these samples and were maintained in serum-free keratinocyte growth medium (Clonetics, San Diego, CA) as described for HENs[6,7] and for HECs.[7,20]

Cell Implantation in Nude Mice

Two- to 3-month-old nude mice were used for HEN/HEC implantation. Barrandon's method[21] was modified as described.[8] Briefly, silicone sheets with freshly attached monolayers of passage 1 cells were implanted into nude mice. For implantation, a skin flap was produced by a v-shaped skin incision in the rear back of the nude mouse. A first silicone sheet was placed on the exposed musculature, and then the silicone sheet to be implanted, with cells facing up, was placed on the first sheet. The implants together with the skin flaps were recovered. These specimens were fixed with 4% paraformaldehyde and embedded in paraffin, or they were snap-frozen in liquid nitrogen-prechilled isopentane and stored at −70 C.

Mucin Staining

To examine the CC mucosecretory differentiated phenotype, cultured monolayer cells and paraffin-embedded stratified sections were stained with Alcian blue at pH 2.5.[8]

Antibodies

Five CKs were examined in this study with six mouse anti-CK monoclonal antibodies. Table 1 summarizes previously observed phenotypes of CCs and RCs in normal endocervical epithelium for the five CKs, including results with monoclonal antibodies used in this study. The following have been consistently observed: CCs express CK7, CK18, and CK19, but do not express CK10/11 and CK13; RCs react with CK13 monoclonal antibody, KS/A3 (Sigma Chemical Co., St. Louis, MO) and CK19, but do not express CK7 and CK10/11.[12-18] Therefore, CK7 is a marker CK specific to endocervical CCs. The monoclonal antibodies LDS-68 (Sigma), K8.60 (Sigma) and Ks19.1 (ICN Immunobiologicals, Lisle,

Table 1. *Cytokeratin Phenotypes of Columnar and Reserve Cells in the Endocervical Epithelium*

CK	MAb	Refs.	CCs	RCs
CK7		13,15	+,+/−	−
CK10/11		12,13,15,18	−	−
CK13	KS-1A3	14	(−)	+
CK18	CK2	13	+	−
CK18		15	+	(+)
CK18	KS-B17.2	14	+	(−/+)
CK19		12,13,15,18	+	+

MAb = monoclonal antibodies also used in this study. +/− = heterogeneously positive for ref. 15. (−) = no staining was usually detected. (+) = weakly positive staining. (−/+) = some weakly positive staining.

IL) were used in this study to detect CK7, CK10/11 and CK19, respectively. In previous publications, opinions have been divided regarding the presence of CK13 and CK18 in RCs.[12–15,18] To experimentally distinguish the CC-like and RC-like HEN CK phenotypes, we used one CK13 monoclonal antibody, KS-1A3, and two CK18 monoclonal antibodies, CK2 (Boehringer Mannheim, Mannheim, Germany) and KS-17.2 (Sigma), that had been used previously (Table 1). RCs, but not CCs, had generally tested positive for CK13 with monoclonal antibody KS-1A3.[14] Alternatively, CCs but not RCs, generally tested positive for CK18 with monoclonal antibodies CK2[13] and KS-17.2.[14]

Cytokeratin Staining

Indirect immunofluorescence assay for CK was performed and staining evaluated as described.[7,8] The cultured cells and cryostat-sectioned frozen specimens were fixed in −20 C methanol, rinsed in acetone, and air-dried. The paraffin-embedded sections were deparaffinized, rehydrated, and treated with 0.25% trypsin (Sigma) at 37 C.[8] The cells and sections were incubated with the required dilutions of primary monoclonal antibodies at 4 C overnight. Goat anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate was employed as the second antibody. For negative controls, the primary antibodies were replaced by phosphate-buffered saline. The antibodies used in this study performed adequately in paraformaldehyde-fixed and paraffin-embedded implantation tissues, as the results obtained were the same as those for frozen tissue.

Results

Morphological Differentiation of Human Endo- and Ectocervical Cells in Vitro *and* in Vivo

Primary HENs and HECs from five different donors were passaged after 2 to 3 weeks in culture. The cells could be passaged at low seeding densities (2 to 4 × 10^3 cells/cm^2) five times for HENs and seven times for HECs before senescence. HENs had two different epithelial morphologies in keratinocyte growth medium, pleomorphic cells and keratinocytelike cells. The pleomorphic epithelial cells were the first outgrowth recognized 4 to 7 days after initiation of primary cultures (Figure 1A, arrow). These cells were also morphologically distinct from HECs, which were continually composed of only typical polygonal keratinocytes (Figure 1D). Within 7 to 12 days of initiation of HEN primary cultures, keratinocytelike cells were observed (Figure 1A, arrowheads), adjacent or close to the initial colonies of pleomorphic cells. After passage, the morphological features varied and depended on the passage number. Cells with a fibroblastic morphology were not found in HEN and HEC cultures grown in serum-free keratinocyte growth medium (Figure 1).

Monolayers of passage 1 HENs were implanted beneath skin-muscle flaps in nude mice. As previously described,[8] these reproducibly formed a stratified epithelial structure similar to that of immature cervical squamous metaplasia 8 days after implantation (Figure 1C). Moreover, within 4 days of implantation, HENs were stratified into double layers of cuboid cells attached to the adjacent mouse tissue (Figure 1B), indicating that the reconstructed epithelium was derived by proliferation of the implanted single HEN monolayer. In contrast to the HEN implants, HEC implants formed a well-differentiated stratified squamous epithelium within 8 days of implantation (Figure 1E). These results exclude the possibility that squamous metaplasia may be formed by lateral migration of the ectocervical basal keratinocytes. Whereas it is possible that the silicone sheets affect HEC differentiation, the unrestricted supply of essential factors was apparent from the absence of the frequent detachment of sheets and the remarkable resemblance of the HEN and HEC implants to *in situ* squamous metaplasia and ectocervical epithelium, respectively, strongly suggesting the fidelity of this *in vivo* system.

Mucin Production of Cervical Cells in Vitro *and* in Vivo

In the HEN primary cultures, Alcian blue specifically stained all the pleomorphic epithelial cells (Figure 2A), indicating mucin production phenotype of CCs. The frequency of the mucin-positive cells decreased with early passage (Table 2, Figure 2). HEN implants showed sporadic suprabasal cells with the vacuolated cytoplasm, suggesting glandular cell differentiation (Figure 1C, arrowhead). Alcian blue specifically stained these cells (Figure 2C, arrowheads). In contrast to HENs, HECs were negative for the mucosecretory phenotype, by morphology *in vivo* (Figure 1E) and by Alcian blue staining *in vitro* (Figure 2D) and *in vivo* (Figure 2E).

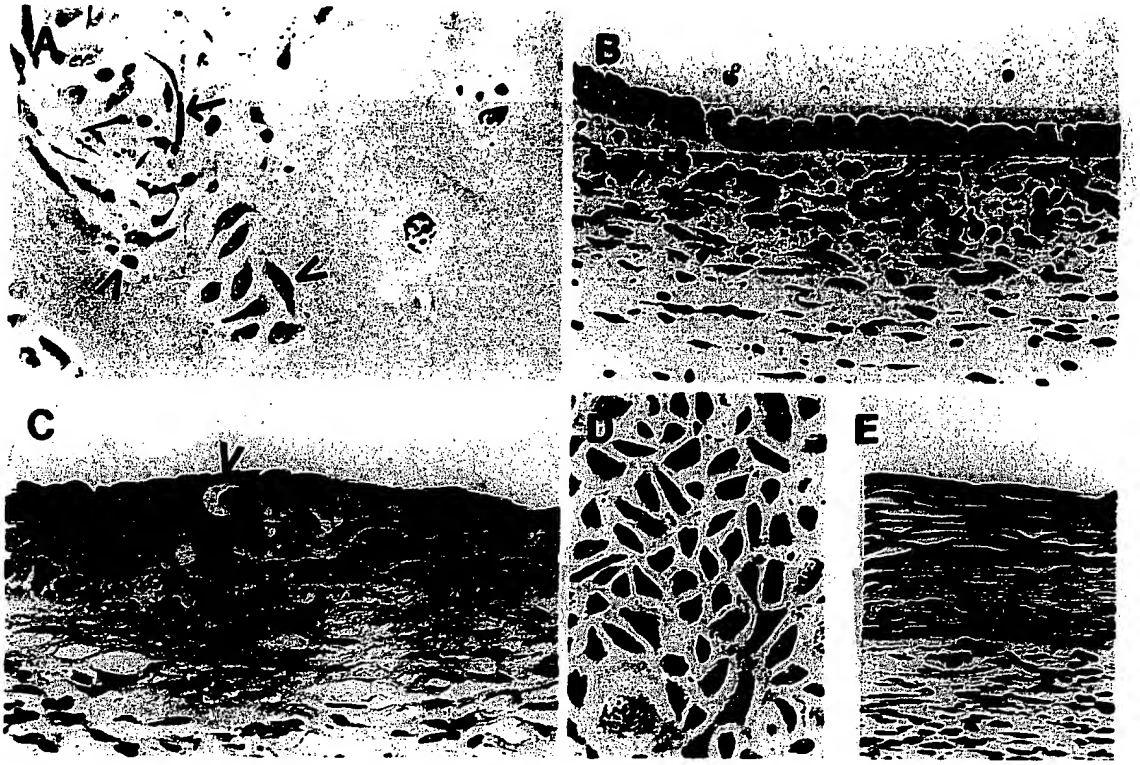

Figure 1. *Morphological differentiation of HENs and HECs in vitro and after implantation in vivo.* **A**: *primary HENs; arrow and arrowheads indicate pleomorphic and keratinocytelike HENs, respectively.* **B**: *implanted passage 1 HENs, 4 days after implantation, H&E staining.* **C**: *implanted HENs, 8 days after implantation, H&E staining; arrowhead indicates a vacuolated cytoplasm in suprabasal layer.* **D**: *primary HECs.* **E**: *implanted passage 1 HECs, 8 days after implantation, H&E staining. Original magnifications:* **A**, **D**, *and* **E** = 200×; **B** *and* **C** = 288×.

Cytokeratin Expression of Endocervical Cells

The CK characteristics of the CC phenotype were also found in the primary HEN that originate as a proliferation of pleomorphic cells. In addition to mucin, these cells expressed the CC markers, CK7 (Figure 3A), and CK18 (Figure 3, H and J). Some of the cells expressed CK13 (Figure 3D). In contrast, all the keratinocytelike cells, that were observed after the initial outgrowth of pleomorphic cells, expressed the CK13 RC marker (Figure 3E). Interestingly, these keratinocytelike cells did not express CK7 (Figure 3B). Early passages of HENs heterogeneously expressed CK7; CK7 was again expressed only in cells which exhibited CC-like cell morphology (Table 2. Figure 3B). The proportion of CK7-positive CC-like cells decreased (Table 2) in later cultures. In addition, the abundance of CC-like cells compared with RC-like cells increased such that the late passage HENs were composed mostly of these CK7-expressing CC-like cells. Both cell types continued to stain for both CK18 antibodies during serial cultivation (Table 2).

In the HEN implants, CK18 was expressed uniformly in the basal layer and heterogeneously in the suprabasal layers (Figure 3L). HENs did not express CK10/11 in *in vivo* implants (Figure 3N) or *in vitro* (Figure 3M), whereas they homogeneously expressed CK19 in both growth conditions (Figure 3, P and O). CK13 was homogeneously expressed throughout all the layers of the HENs *in vivo* (Figure 3G). CK7 was expressed, and mucin was produced, in scattered cells and only in suprabasal layers (Figures 3C and 2C). This is consistent with the mucin-negative, CK7-negative, RC-like HENs being the direct precursor basal cells for experimental squamous metaplasia.

Cytokeratin Expression of Ectocervical Cells

A distinct CK expression pattern was found for HECs, as compared with HENs, propagated *in vitro* and in *in vivo* implants. All ectocervical cells failed to express CK7, consistent with the HEC/HEN pattern

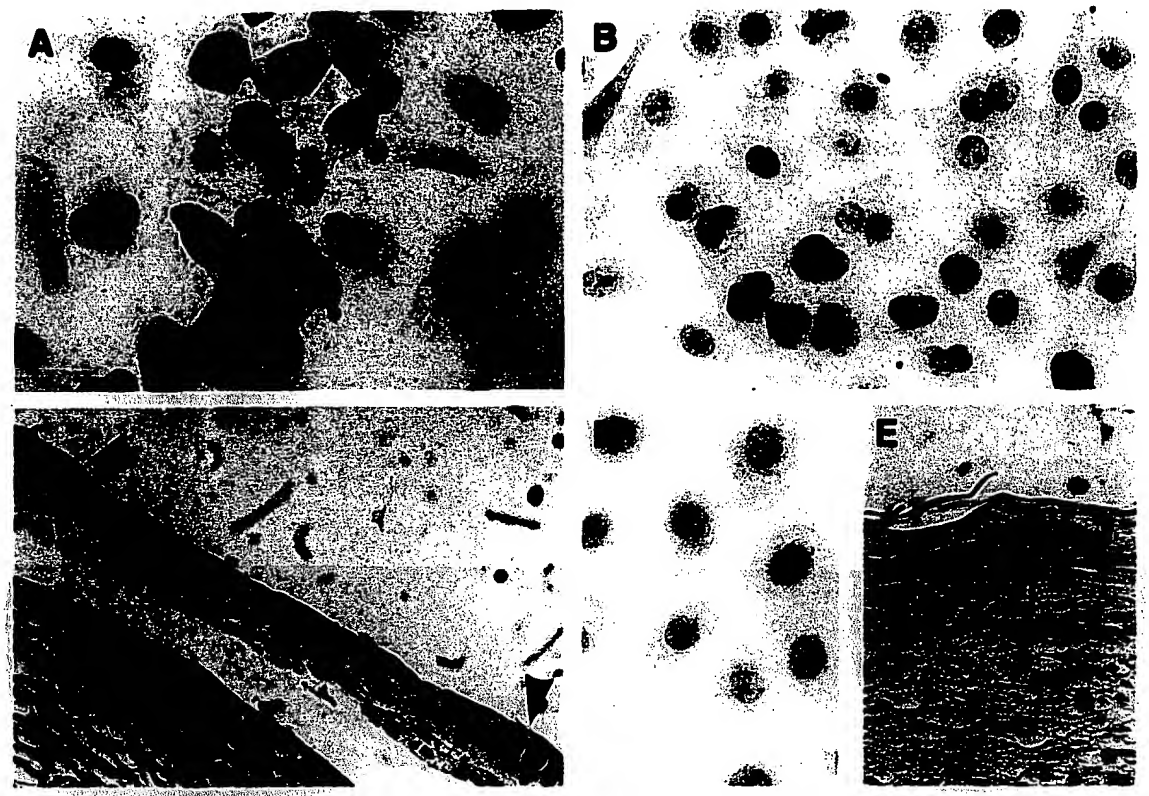

Figure 2. *Alcian-blue staining for mucin production of HENs and HECs in vitro and after implantation in vivo.* **A:** *primary culture pleomorphic HEN (mucin-positive).* **B:** *passage 2 HENs; note that mucin-positive cells decreased with passage.* **C:** *HENs 8 days after implantation; note that some superficial cells were positive for mucin production (arrowheads).* **D:** *passage 2 HECs (mucin-negative).* **E:** *HECs (mucin-negative) 8 days after implantation. Original magnifications:* **A, B,** *and* **D** = 560×; **C** *and* **E** = 200×.

(Table 2) expected from *in situ* observations[15] for this marker. As well as the CK7 and mucin patterns *in vitro* and *in vivo*, the HEC CK13 pattern *in vitro* pattern (Figure 4C) was the same as for the morphologically identical HEN RC-like cells, but not the dissimilar CC-like cells. However, neither CK18 antibody could stain HEC *in vitro* or in any of the cells of the implants (Figure 4, E and F; Table 2). All the other *in vivo* CK phenotypes of HECs were also distinct from those of HENs. For the HEC implants, there was expression of CK10/11 and the expression patterns of CK13 and CK19 varied in relation to the state of squamous differentiation (Table 2). CK10/11 was expressed *in vivo*, although not *in vitro*; expression of this keratinization marker was confined to random clusters of suprabasal layer cells (Figure 4B). CK13 showed a strong expression pattern in the parabasal and intermediate layer cells (Figure 4D); some basal and superficial cells were also stained. CK19, that had been stained for all HENs, was also expressed homogeneously in HECs *in vitro* and in almost all basal cells in *in vivo* implants; however, only some

Table 2. *Cytokeratin Expression and Mucin Production of HENs and HECs* in Vitro *and* in Vivo

		HENs *in vitro*						HENs *in vivo*		HECs *in vivo*				
		Primary		Early		Late				HECs				
CK/mucin	MAb	CC-like	RC-like	CC-like	RC-like	CC-like	RC-like	B	SB	*in vitro*	B	PB	I	S
CK7	LDS-68	+	–	+	–	+/–	–	–	+/–	–	–	–	–	–
CK10/11	K8.60	–	–	–	–	–	–	–	–	–	–	+/–	+/–	+/–
CK13	KS-1A3	+/–	+	+/–	+/–	+	+	+	+	+	+/–	+	+	+/–
CK18	CK2	+	+	+	+	+	+	+	+/–	–	–	–	–	–
CK18	KS-B17.2	+	+	+	+	+	+	+	+/–	–	–	–	–	–
CK19	K19.1	+	+	+	+	+	+	+	+	+	+	+/–	–	–
Mucin		+	–	+/–	–	+/–	–	–	+/–	–	–	–	–	–

Early = passage 1 and 2; Late = 1 and 2 passages before senescence (passage 3 and 4); MAb = monoclonal antibody; B = basal layer; SB = suprabasal layer; PB = parabasal layer; I = intermediate layer; S = superficial layer; + = homogeneously positive; +/– = heterogeneously positive; – = negative.

Figure 3. *Immunofluorescence detection of CKs of HENs* in vitro *and after implantation* in vivo. **A** *to* **C** = *CK7*. **D** *to* **G** = *CK13*. **H** *to* **L** = *CK18* (*monoclonal antibodies*: **H** *and* **I**, *CK2*; **J** *to* **L**, *Ks-17.2*). **M** *and* **N** = *CK10 + 11*. **O** *and* **P** = *CK19*. **A**, **B**, **D** *to* **F**, **H** *to* **K**, **M**, *and* **O** = in vitro. **C**, **G**, **L**, **N**, *and* **P** = in vivo. **A**: *primary HENs*; **B**: *passage 2 HENs*; **D**: *primary HENs*; **E**: *passage 1 HENs*; **F**: *passage 2 HENs*; **H** *to* **K**: *passage 2 HENs*; **M** *and* **O**: *passage 2 HENs*. *Original magnification, 288×*.

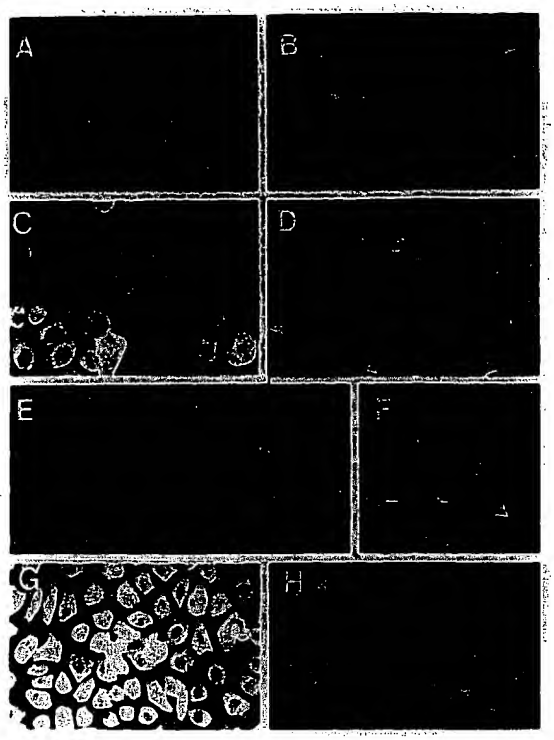

Figure 4. *Immunofluorescence detection of CKs of HECs* in vitro *and after implantation* in vivo. **A** *and* **B**: *CK10+ 11.* **C** *and* **D**: *CK13.* **E** *and* **F**: *CK18 (monoclonal antibody Ks-17.2).* **G** *and* **H**: *CK19.* **A, C, E,** *and* **G**: *passage 2 HECs* in vitro. **B, D, F,** *and* **H** = in vivo. *Original magnification, 288×.*

suprabasal and no intermediate or superficial layer cells were positive (Figure 4H).

Discussion

The majority of premalignant and malignant lesions of the cervix arise in the transformation zone, the border of the ecto- and endocervices, in which the endocervical simple epithelium is transdifferentiated into squamous metaplastic epithelium.[2,3,22] The precise mechanism of transdifferentiation remains poorly understood, and the confusion involves the exact cellular origin of metaplasia. Recently, it has been inferred from morphological and/or immunohistochemical *in situ* observations[2,3,10–12,14,19] that the squamous metaplastic cells are generated by transformation of RCs. Several hypothetical sources have been proposed as the cellular origin of RCs: embryonal crests of urogenital origin, basal keratinocytes of fetal squamous epithelium, and stromal cells.[2,3] The results, with the HEN culture/implantation system, which reproducibly gave a stratified epithelium in nude mice (Figure 1), suggest a possible pathway for the histogenesis of cervical squamous metaplasia. The cervical squamous metaplasia may originate by a unique route, which involves CC⇆RC transformation as an intermediate step. Others have suggested the CC origin of RCs. Based on studies of CKs, Weikel et al[13] proposed that RCs are derivatives of, rather than precursors for CCs. Based on staining for CKs in individual CCs, Franke et al[23] proposed that these individual cells may be the precursors of squamous metaplasia. The alternative possibility would be that RCs are the direct precursors of squamous metaplasia. Several reports support the view that the RCs originate from the epithelia between the endocervical columnar cells and the basement membrane.[19,24]

The expression patterns of CKs in CCs have been fairly consistent in previous publications: they are positive for CK7, 18, and 19, but are negative for CK10/11 and 13. In addition, RCs express CK19, but do not express CK7 and 10/11.[12–15,18] The discrepancies as to whether CK13 is expressed in RCs can be explained by different CK13 antibodies recognizing different epitopes or exhibiting varying affinities for their respective antigens.[14,15] Whereas CK13 is a differentiation marker, the monoclonal antibody KS-1A3 is a special marker for both squamous differentiating and nondifferentiating keratinocytes and endocervical RCs.[14]

We examined whether the epithelium produced in the HEN culture/implantation system, that displayed experimental cervical squamous metaplasia, could arise by proliferation of either CCs or RCs.[6–8] *In situ*, mucus-secreting CCs have been observed to replicate.[24] As the primary cultures could similarly have originally been either CCs or RCs,[6–8] we analyzed the HEN CK phenotypes. Because the HENs at all passages expressed CK7, the primary outgrowths were unlikely to have originated from CK7-negative RCs. More likely, the origin of these CK13-heterogeneous, CK7-positive cells was CCs (Tables 1 and 2). With advanced time and passages *in vitro*, the HENs developed a more CK7-negative, CK13-positive RC phenotype. This was especially applicable to the RC-like cells that appeared to develop from the CC-like cells (Table 2; Figure 1). Whereas the possibility that RCs exist as a minor population in the original culture cannot be excluded, these data support the suggestion that the HENs are bipotential in nature and can undergo differentiation reversibly to give rise to CCs or RCs. This bipotential phenotype may also explain the CK18 expression of the RC-like HENs (as detected by CK2 antibody) and the CK13 expression of the CC-like HENs (Table 2).

Both HENs and HECs consistently were reconstructed into stratified layers of epithelium upon im-

plantation *in vivo*. The HENs reproducibly exhibited the squamous metaplastic phenotype, including the ability to form a stratified epithelium. These were very similar to the corresponding cervical squamous metaplasia *in situ*, in morphological, mucin production, and CK expression patterns. Also, many of the phenotypes of the HECs were retained and were displayed in greater, relevant detail in the implants. The expression patterns of the CK13 and CK19 that are also expressed in RCs, ectocervical epithelium, and squamous metaplasia,[12,14,15,19] were clearly distinct for HENs and HECs. The *in vivo* HEN CK expression pattern suggests that the RC-like cells are the origin of squamous metaplasia. The RC-like cells *in vitro* are also more similar than the CC-like cells to the basal layer of the HEN implant; the basal layer is the normal site of RCs and is more directly derived from the implanted monolayer. The suprabasal HENs are more similar to CCs in their heterogeneous mucin production. Thus HENs, as opposed to HECs, are clearly linked to metaplasia by morphology and mucin and CK expression.

Another endocervical CC phenotype of interest is retained by the suprabasal HENs *in vivo*—CK7 expression. CK7 expression is also observed in CIN lesions.[15] The large majority of CIN are induced by human papillomavirus (HPV),[25] and induction may be selective for a specific phenotype of endocervical cells, which could include CK7 expression. We have found that HPV 16-immortalized HENs develop into *in vivo* lesions with high-grade CIN morphological and mucin-expressing phenotypes.[8] Moreover, CK7 was expressed in these CINIII/carcinoma *in situ* lesions, and this expression was stronger (data not shown) than in the implants of normal HENs (Figure 3C). Furthermore, in *in situ* tissues, there was stronger expression of CK7 in CINIII than in CINI and II.[15]

There has been a concerted effort to develop an experimental model for studying the dynamics of cervical carcinogenesis. The convincing epidemiological and etiological associations of high-risk HPVs with high-grade CINs and cervical carcinomas has heightened and focused interest in the culture of human cervical epithelial cells.[7,8,26] Squamous metaplasia and HPV infection are thought to play central roles in the pathogenesis of CINs.[2,3,15,18,19,25,27] We believe our experimental *in vitro*/*in vivo* system, applied to the normal endocervical cells, as well as our novel HPV16-immortalized endocervical cells,[7] will be a powerful tool for analyzing the interaction between endocervical squamous metaplasia and HPV gene expression in the development of cervical carcinomas.[8]

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